D.U.Quark

Volume 2
Issue 1 *Fall 2017*

Article 2

6-25-2017

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D.U.Quark 2017(2) pgs. 17-22

Published June 25, 2017

Peer Reviewed Articles

Courtesy of Dr. O'Donnell's Advanced Topics course on Regulated Protein Degradation

Abstract. How does cell signaling in response to extracellular stressors impact the trafficking of membrane proteins? In particular, the TORC1 complex plays a key role in this process and while some details of this system have reported, in a recent Journal of Biological Chemistry publication, Andre's group has revealed new details of this pathway focusing on the general amino acid permease Gap1 as a model cargo. Andre et al. describe a novel and distinct pathway wherein ubiquitylation and downregulation of Gap1 is regulated not only by amino acid-induced activation of TORC1, but also by numerous sources of TORC1 inhibition and cellular stress.

An incomplete picture. Situational control of plasma membrane (PM) proteins is critical for cell survival, and studies of regulated protein trafficking in Saccharomyces cerevisiae have been instrumental in elucidating the mechanisms underlying these events. The general amino acid permease Gap1 is a longstanding model for these studies and has provided key insights into regulation of transporters, particularly in the context of ubiquitin-dependent trafficking (1). Prevalent at the membrane under poor nitrogen (N) conditions, Gap1 undergoes Rsp5-mediated ubiquitylation followed by transport to the vacuole for degradation in response to increased internal amino acid levels (2). As Gap1 and many other transporters themselves do not possess the necessary [P/L]PxY motifs required for interaction with Rsp5 (3), arrestin-related trafficking adaptors (ARTs) act as mediators in these protein trafficking pathways (4). Earlier studies from the Andre and Kaiser labs show that Bul1 and Bul2, two ART family members, control Gap1 trafficking; Buls are phospho-inhibited by the Npr1 kinase, which is negatively regulated by TORC1, and bind to 14-3-3 proteins under poor N conditions inhibiting Bul-mediated endocytosis of Gap1, however this phospho-inhibition is relieved when TORC1 is activated by increased amino acid levels and Npr1 is inactivated (2). What remains unclear is how Gap1 trafficking is regulated following TORC1 inhibition or under stress conditions.

Gap1: Insights into complex regulatory trafficking networks. Cells grown in the presence of a poor N source will maintain Gap1 as a stable and active protein at the cell

surface. In response to the addition of a good N source, like ammonium (Am), TORC1 is activated and the Bul1 and Bul2 adaptors are no longer phosphorylated. In this form, Buls recruit Rsp5 to Gap1 and Gap1 is ubiquitinated and trafficked to the vacuole to down-regulate its activity (2,5). Based on this model, one would predict that inhibition of the TORC1 complex should prevent N-induced, endocytosis as the Bul adaptors would maintain their phospho-inhibited state (6). Surprisingly, Andre et al. show that Gap1 is strongly downregulated following Am treatment in the presence of the TORC1 inhibitor rapamycin (Rapa). In fact, Rapa alone is sufficient to trigger Gap1 trafficking to the vacuole and does so in an Rsp5-dependent manner. These results demonstrate contrasting functions for TORC1, as the complex promotes Gap1 ubiquitylation when internal amino acid levels are high (2), yet TORC1 clearly plays a key role in maintaining Gap1 at the plasma membrane in poor N conditions (6). Andre's group built on previous work demonstrating a role for the arrestin-like yeast proteins Aly1 and Aly2 in regulation of Gap1 trafficking (7), showing that while the Bul and Aly adaptors are sufficient to fully protect against Rapainduced down-regulation; In bul1,2Δ aly1,2Δ there is no ubiquitinated Gap1 and it is stable at the PM in response to Rapa. From this work it is clear that the regulatory system is more complex than previously predicted, with Aly and Bul proteins acting synergistically to regulate Gap1. When stimulated, the TORC1 complex activates Sit4-mediated dephosphorylation of the Bul proteins,triggering their disassociation from the 14-3-3 proteins, and allowing them to mediate Rsp5-dependent endocytosis of Gap1 (2). Surprisingly, the authors propose that the Bul/Aly proteins retain their phosphorylation and association with 14-3-3 proteins in response to Rapa, suggesting the inhibition to be situational and incomplete. This is in contrast to other models of 14-3-3 inhibition of ARTs (13).

A tale of two tails. Utilizing systematic mutational analysis, the authors identify two regions of Gap1's N-terminal cytosolic tail, near the K9 and K16 Ub-accepting residues, critical for dephosphorylated Bul/Aly interaction. Importantly, these regions do not seem to play a role in Rapainduced regulation, strongly suggesting that the phosphorylated forms of Bul/Aly act on different regions of Gap1. Andre's group also identifies regions in Gap1's C-terminal tail critical for its Rapainduceddown-regulation. This surprising and intriguing finding is the first evidence that ARTs can recognize two distinct regions of a transporter depending on their phosphorylation status. Excitingly, other known noxious stressors (heat shock, oxidative and alcoholic stress) (8,9) seem to illicit the same pattern of regulation by the Bul/Aly adaptors as Rapa treatment (6), broadening the potential scope and impact of this regulatory system.

The authors further investigate the TORC1 subunit Tco89, a previously reported connection to Rapa hypersensitivity (10). Critically, tco89Δ strains show growth defects on poor N mediums, such as citrulline or proline, consistent with defects in Gap1 function. Supporting this finding, Gap1 is constitutively internalized in a Ub-dependent

manner in tco89Δ cells which further solidifies the connection to the Rapa-induced regulation. Investigating the possibility that other transporters may be regulated in an analogous fashion to Gap1, the authors show that three other permeases (Can1, Lyp1, and Fur4) are also internalized in response to Rapa and noxious stressors. Importantly, their results show Can1 and Fur4 are protected from down-regulation in rsp5 (npi1) mutants, but not in their Bul/Aly quadruple mutant, potentially broadening this regulatory network to involve other ARTs.

Closing the Gap. There now appear to be two distinct regulatory pathways utilizing ART proteins to control Gap1 localization. Under rich N conditions or high levels of internal amino acids, Gap1 is down-regulated following dephosphorylation of the Bul/Aly adaptors in response to TORC1 stimulation (Fig 1A). While these ARTs are normally phospho-inhibited under poor-N conditions, allowing Gap1 to remain at the PM (Fig 1B), TORC1 inhibition or stress triggers a new response; The Bul/Aly adaptors maintain their phosphorylation and binding to 14-3-3 proteins, typically consider inhibitory, and recognize the C-terminal tail of Gap1 to control its endocytosis (Fig 1C). Importantly, the TORC1 subunit Tco89 is a key player in this new regulatory scheme. Though the authors demonstrate the importance of Tco89, particularly in N-poor conditions, more remains to be elucidated regarding its specific inhibitory role of the ART adaptors. Perhaps this subunit typically acts to inhibit ART binding to the C-terminal tail of Gap1, a role that could then be disrupted following stress or TORC1 inhibition by Rapa. The authors also raise the interesting possibility that, despite established connections between Art1 and cycloheximide-induced endocytosis of the Can1 arginine permease (12), other ARTs may be involved in Rapa-induced down-regulation. Indeed, the precise mechanisms behind arrestinmediated stress responses remain undefined. One proposed model, considered unlikely by the authors, features arrestins recognizing permease misfolding triggered by such stressors. Another possibility is raised that multiple pathways are stimulating the ARTs and controlling Rsp5 activation in these conditions. Given the exquisite phosphor-regulation of these proteins – with over 25 phospho-sites identified in Art1 and Aly1 by MS analyses (14) – this seems a likely scenario. Different combinations of phosphorylation might fine tune arrestin function and response to different stress conditions. These open questions demonstrate the as-yet incomplete understanding of the regulatory network controlling yeast membrane protein trafficking in response to select environmental cues.

Figure 1

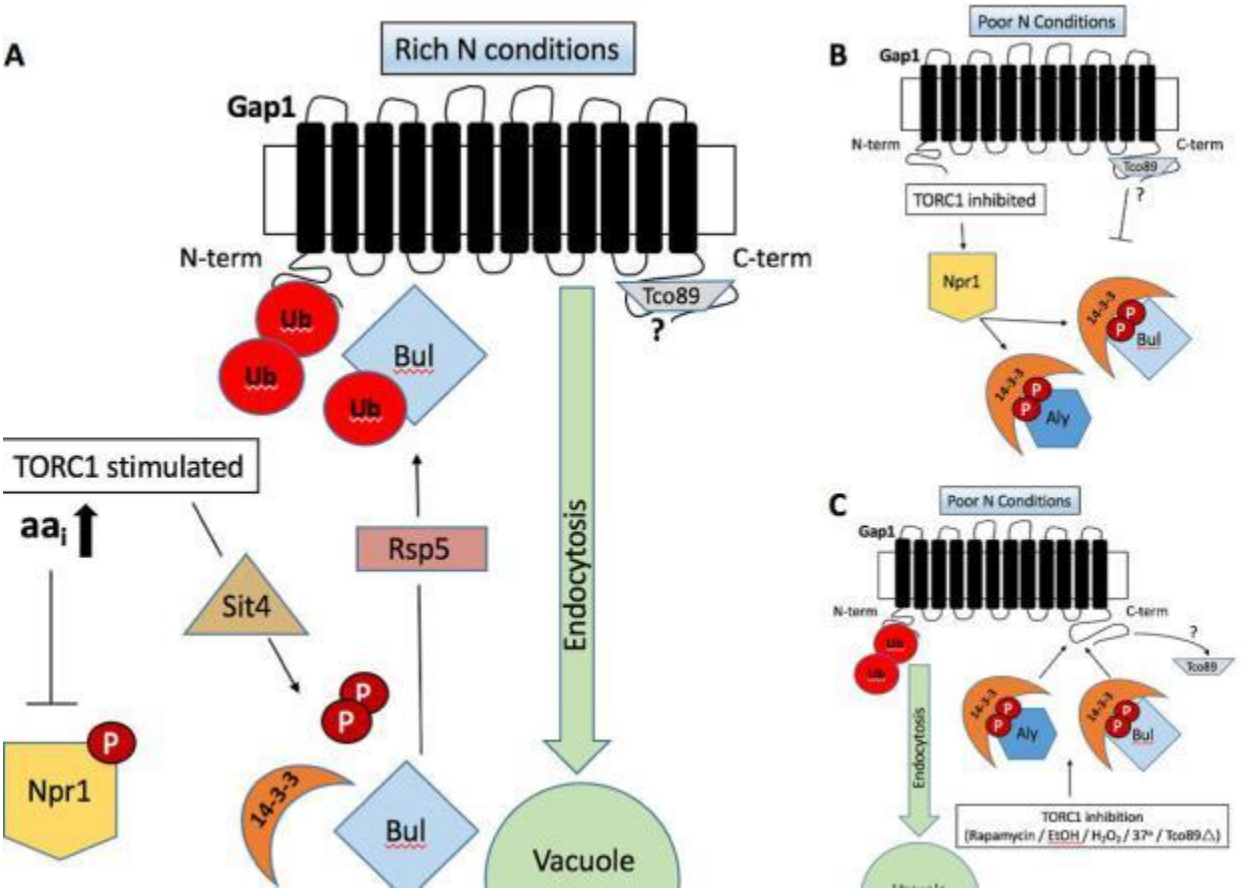

Figure 1. (A) When TORC1 is active and stimulated by high levels of internal amino acids, Npr1 is phospho-inhibited and the Bul proteins can be dephosphorylated by Sit4 leading to their disassociation from 14-3-3 proteins and mono-ubiquitylation. This in turn allows them to act via N-terminal regions of Gap1 to allow its Rsp5-mediated ubiquitylation, endocytosis, and transport to the vacuolar lumen. (B) Under poor N conditions, Npr1 kinase is active and phosphorylates the Bul and Aly adaptors which can then be bound by 14-3-3 proteins, perhaps preventing their ability to bind to the N-term tail of Gap1. Gap1 is not ubiquitylated under these conditions. This may be due to the adaptors' lack of access to the N-term region of Gap1 or even the TORC1 subunit Tco89 somehow inhibiting their ability to bind to the C-terminal region. (C) When TORC1 is inhibited by rapamycin, stress, or loss of Tco89, the Aly and Bul proteins, still phosphorylated and bound to 14-3-3 proteins, are able to act through C-terminal regions of Gap1 to allow Rsp-5 mediated ubiquitylation of lysine residues near the N-terminus (K16 for Aly 1,2 and both K9 and K16 for Bul1,2). This in turn triggers endocytosis of Gap1 and its transport to the vacuolar lumen.

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Bowman, Ray. "The General Amino Acid Permease Gap1 is Regulated Differentially by TORC1 Activation and Inhibition." *The D.U.Quark,* 2, 1 (2017): 17-22. https://duquark.com/2017/06/25/the-general-amino-acid-permease-gap1-is-regulated-differentially-by-torc1-activation-and-inhibition/